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(54) Title: ANALYTICAL METHOD COMPRISING ADDITION IN TWO OR MORE POSITIONS AND A DEVICE AND TEST KIT THEREFOR

$$LZ_m \quad . \quad . \quad . \quad LZ_n \quad . \quad . \quad . \quad LZ_1 \qquad DZ$$
$$\text{- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -} \quad > \qquad (I)$$

(57) Abstract

A method and a device and test kit, respectively, for determination of an analyte in a sample in a flow matrix by means of a transport flow of one or more biospecific affinity reactants, at least one of which is analytically detectable (Reactant*) and one is firmly anchored in the matrix (Reactant I), have the characterizing features that: A. the flow matrix has at least two application zones for liquid (I) wherein a) $LZ_n$ is an application zone for liquid, and n is the position of the application zone $LZ_n$, b) m is the total number of application zones in which flow is initiated (m ≥2), c) one $LZ_n$ is an application zone for sample ($LZ_n$·S) and one $LZ_n$ is for Reactant* ($LZ_n$··R*) with n'' ≥n', d) ————— > is the direction of the flow, e) DZ is the detection zone, and B. flow is initiated by adding liquid to each zone $LZ_m$ .. $LZ_n$ .. $LZ_1$ in such a way that liquid$_{n+1}$, added to the application zone $LZ_{n+1}$, is transported through the matrix immediately after liquid$_n$, added to the nearest downstream application zone $LZ_n$.

# ANALYTICAL METHOD COMPRISING ADDITION IN TWO OR MORE POSITIONS AND A DEVICE AND TEST KIT THEREFOR

## Technical field

5   The invention relates to a method for determination of an
analyte in a sample by use of biospecific affinity
reactants (Reactant 1, Reactant 2 etc.), one of which is
analytically detectable (Reactant*) and one is firmly
anchored in a detection zone in a transport flow matrix
10  (Reactant I). The sample (analyte) is transported by a flow
in the matrix from one application zone for liquid (LZS)
containing the analyte (sample) and/or a buffer, to the
detection zone (DZ), in which Reactant I is firmly
anchored. At the same time as the sample is transported in
15  the matrix the soluble reactants, including Reactant*, are
also being transported. In the detection zone Reactant* is
captured in an amount which is related to the amount of
analyte present in the sample. To achieve this, Reactant*
is chosen so that it may bind biospecifically directly to
20  Reactant I or indirectly via one or more added biospecific
affinity reactants (including the analyte). The amount of
analyte is then determined from the amount of Reactant*
bound in the detection zone. The transport flow may contain
zones, in which different biospecific affinity reactants
25  (e.g. Reactant*, but not analyte) have been applied in
advance (predeposited) in order to be dissolved and
transported along with the flow towards the detection zone.

By reactants (including the analyte), exhibiting
30  biospecific affinity (bioaffinity reactants), are meant
individual members in the reactant pairs: antigen/hapten -
antibody; biotin - avidin/streptavidin; two complementary
single chains of nucleic acid etc. As antibodies antigen
binding antibody fragments such as Fab, $F(ab)_2'$, single
35  chain Fv antibodies (scFv) etc. are considered. Relevant
reactants do not have to be naturally occurring but can
also be synthetically produced molecules/binders.

The type of test methodology in question has previously
been used primarily for biospecific affinity reactants
where at least one part of an employed reactant pair has
5    exhibited protein structure, in particular in connection
with so called immunochemical determination processes.

The biospecific affinity reactions are primarily performed
in aqueous media (e.g. water).
10

The technique in question is well known and has often been
applied to so called test strips, where the strip has
functioned as a flow matrix. The flow has been initiated in
the zone to which the sample has been added (LZS). The flow
15   has often been lateral, i.e. parallel to the surface of the
matrix, or of other types, e.g. in depth in the matrix.

The test protocols have been of so called inhibition type
(competitive) or non-inhibition type (non-competitive,
20   sandwich). See e.g. Behringwerke US 4,861,711; Unilever WO
88/08534; Abbot US 5,120,643; Becton Dickinson EP 284,232
and US 4,855,240; Abbot/Syntex US 4,740,468; Pharmacia AB,
WO 96/22532, etc.

25   In this context one often mentions simultaneous and
sequential methods (protocols) regarding certain reactants
(especially analyte and Reactant*). In simultaneous
variants analyte (sample) and the relevant reactant, e.g.
Reactant*, are transported simultaneously into the
30   detection zone. Simultaneous variants may be obtained, if a
sample is pre-incubated/mixed with Reactant* or if
Reactant* has been predeposited in the sample application
zone or in a zone downstream of the sample application zone
but before the detection zone. In sequential variants the
35   analyte (sample) is transported before a reactant, e.g.
Reactant*, into the detection zone. Sequential variants may
be obtained, if the relevant reactant, e.g. Reactant*, is
added to the same application zone as the sample after the

sample (analyte) has been transported out of the zone. A
variant of sequential methodology is discussed in US
4,855,240 (Becton & Dickinson). As an alternative to the
sample (analyte) being transported before "tracer"
5  (=Reactant*) in the same transport flow, US 4,855,240
describes separated transport flows, in which the time of
transport is regulated such that sample (analyte) reaches
the detection zone before the "tracer" (Reactant*).


10  The term simultaneous tests has often included every
variant, in which sample and Reactant* are pre-
incubated/mixed before being added to a flow matrix or in
which sample is added to a flow matrix, in which Reactant*
is predeposited in the sample application zone or
15  downstream thereof. The term sequential tests has similarly
included every variant, in which Reactant* is added to the
sample application zone after the sample has migrated out
of its application zone. Thus, considerations have not been
taken, concerning if the order of analyte and Reactant* is
20  changed during transport to the detection zone. If not
otherwise stated, this nomenclature is also used for the
present invention, but is now adapted so that there are
several application zones for liquid. This view means that
primarily the initial order is considered, when both
25  analyte and Reactant* are in soluble form, and not the
order in which analyte and Reactant* are transported into
the detection zone.


**Disadvantages with prior art and objects of the invention.**
30  The prior art has often involved practical problems on
automation, primarily because pre-incubation or sequential
addition of sample and reactants have often been required,
often in a certain predetermined order defined by the test
protocol used. The object of the invention is to (a)
35  facilitate automation, (b) avoid sequential addition of
sample and the analytically detectable reactant
(Reactant*), and (c) allow for pre-deposited Reactant* when
using sequential methodology, which relates to analyte and

Reactant*. More general aims are to achieve high quality
test results, preferably with improved sensitivity and
precision than given by previous variants.

5  **The invention**

Surprisingly we have now discovered that if flow is
initiated by almost simultaneous addition of liquid to two
adjacent zones in a flow matrix, the liquid added in the
downstream zone migrates before the liquid which has been
10  added in the upstream zone in direction towards the
detection zone. Our discovery involves that zonewise
migration of liquids may also be obtained if addition of
liquid in an upstream zone is performed after addition of
liquid in the nearest downstream zone. By applying this
15  discovery on the relevant type of analysis methods,
improvements can be obtained regarding the objects stated
above.

A first main aspect of the invention relates to the
20  initially mentioned analysis methods and is characterized
in that

A. the flow matrix exhibits at least two application zones
for liquid arranged substantially adjacent to each other:
25

$LZ_m$ .   .   . $LZ_n$ .   .   . $LZ_1$              DZ

-------------------------------------------->

wherein

30       a) $LZ_n$ is an application zone for liquid, where n is
         the position of the application zone $LZ_n$ (n is an
         integer $2 < n \leq m$)
         b) m is the total number of application zones, in
         which flow is initiated,
35       c) one $LZ_n$ is an application zone for sample ($LZ_n, S$)
         and one $LZ_n$ is application zone for Reactant*
         ($LZ_{n''}, R*$) with $n'' \geq n'$,

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with the exception of n = m and n = 1 for which the zones
$LZ_{n+1}$ and $LZ_{n-1}$, respectively, are lacking).


By the expression "substantially adjacent to each other" is
5 meant that the application zones for liquid are immediately
adjacent to each other or with an intermediate area of
matrix which preferaby is no more than about 2 mm, and
particularly no more than about 1 mm.


10 A liquid added in an application zone may have a tendency
to spread on top of the matrix to parts of the matrix being
outside the zone. For adjacent zones this means that
liquids may be mixed with each other in an undesired way.
To avoid this, physical barriers delimiting two adjacent
15 application zones (zone spacers) are placed. The barriers
should primarily be placed on top of the matrix, but may be
extended down into the matrix without completely quenching
the flow. Delimitation is primarily against an adjacent
zone for application of liquid, but can of course extend
20 around a whole application zone for liquid. Liquid may also
be introduced via pads or material layers applied on the
matrix and from the same or a different material than the
matrix material. In such as case there is no need for zone
spacers.
25

Relevant reactants may be predeposited in an application
zone for liquid ($LZ_n$) or between two such zones. An
application zone for liquid, only intended for transporting
buffering components and/or other components not
30 participating in the biospecific affinity reactions (i.e.
liquid neither containing nor intended for transport of any
reactant or analyte), is called $LZ_nB$ below. An application
zone for liquid ($LZ_n$), where the liquid contains a reactant
or is intended for transport of a reactant, e.g. Reactant*,
35 Reactant 1, Reactant 2 etc., is called $LZ_n..R*$, $LZ_n...R1$,
$LZ_n....R2$ etc. below. If a $liquid_n$ is to transport a
combination of components, e.g. Reactant* and analyte
(sample) the application zone will be common for the

components and will be designated $LZ_{n'}.R2/R1$ etc. For the
combination sample and Reactant*, the application zone will
be $LZ_{n'}.R*/S$ (n' = n''). That $liquid_n$ is intended for
transport of a certain reactant includes that the reactant
5   in question also can be predeposited in the zone $LZ_n$. The
latter includes that the reactant may be predeposited in an
area downstream of the application zone for the relevant
liquid but upstream of the nearest downstream located LZ
$(LZ_{n-1})$, or if n = 1 only upstream of the detection zone (as
10  $LZ_{n-1}$ then is lacking).

By predeposition is meant that a reactant is added in
advance to the matrix and in a way so as not to spread in
the matrix until it is reached by liquid, which has been
15  applied to initiate flow. Predeposition of reactants may be
performed in a way known per se. (See e.g. Behringwerke US
4,861,711; Unilever WO 88/08534; Abbot US 5,120,643; Becton
Dickinson EP 284,232). It is important that arrangements
are made so that the reactant in question is quickly
20  dissolved, when liquid passes through an area, containing
predeposited reactant. In order to achieve quick
dissolution it has been common to incorporate reactants in
substances that as such dissolve quickly. This type of
substances are often hydrophilic with polar and/or charged
25  groups, such as hydroxy, carboxy, amino, sulphonate etc. In
particular there may be mentioned hydrophilic quickly
soluble polymers, e.g. having carbohydrate structure,
simple sugars including mono-, di- and oligosaccharides and
corresponding sugar alcohols (mannitol, sorbitol etc.). It
30  is common practice to first coat the application zone in
question with a layer of the quickly soluble substance, and
then the reactant is applied, optionally followed by one
additional layer of quickly soluble substance. An
alternative way is by incorporating the reactant in
35  particles of quickly soluble material which then are
deposited in the relevant zone of the matrix.

Some of the most important embodiments regarding the
application zones for liquid may be summarized: $2 \leq m \leq 6$;
$n'$ is 1, 2 or 3; $n'' > n'$ or $n'' = n'$; $LZ_{n'}.S$ is the
application zone for sample and optionally also for

5    Reactant* or other reactant; $LZ_{n'+1}$, $LZ_{n'+2}$, $LZ_{n'+3}$, $LZ_{n'-1}$, and
$LZ_{n'-2}$ are application zones for liquids intended for
transport of Reactant* or other reactant or buffer without
reactant as far as allowed by m, n'' and n'.

10   Transport flow through the particular types of matrix may
be achieved by the action of capillary forces, e.g. by
starting with a substantially dry matrix. A sucking body
may be placed at the end of the flow as an aid. Flow,
meaning transport of primarily only dissolved components,

15   may be achieved if an electrical field is applied across
the matrix.

**Test protocols**

By use of the invention reactants and analyte can be made
20   to migrate zonewise as individual components or together in
different combinations towards the detection zone. The
exact sequence of application zones is determined by the
test protocol to be utilized.

25   The invention may be applied to competitive (inhibition) as
well as non-competitive (non-inhibition) test variants
irrespective of if these are simultaneous or sequential
regarding any reactant. Illustrative systems are shown
schematically below in form of the complexes formed. "-"
30   relates to firm anchoring to the matrix, "---" relates to
binding via biospecific affinity. For the sake of
simplicity it has been assumed that reactants used are
monovalent regarding utilized binding sites.

A. Sandwich protocol (non-inhibition)

Reactant I and Reactant* both have biospecific affinity for the analyte. $x$ = number of moles of Reactant I on the matrix and $y$ = number of moles of analyte (= number of

5  moles of Reactant*), bound to Reactant I.


Complex in the detection zone:
Matrix (-Reactant I)$_{x-y}$ (-Reactant I --- analyte --- Reactant*)$_y$

10

Simultaneous variants:
$m = 2$: LZ$_2$R*/S   LZ$_1$B   DZ


Sequential variants:
15  $m = 2$: LZ$_2$R*   LZ$_1$S   DZ.

$m = 3$: LZ$_3$R*   LZ$_2$B   LZ$_1$S   DZ and alternatives where the buffer zone has position 1 or 3.

$m = 4$: LZ$_4$B   LZ$_3$R*   LZ$_2$B   LZ$_1$S   DZ and alternatives where any of the buffer zones is placed in position 1.

20  $m = 5$: The same sequence as for $m = 4$ with the exception that an extra buffer zone is placed in position 1.


B. Sandwich protocol (non-inhibition):

Reactant I exhibits biospecific affinity for Reactant II.
25  Both Reactant II and Reactant* have biospecific affinity for the analyte. $x$ = number of moles of Reactant I on the matrix, $y$ = number of moles of analyte (= number of moles of Reactant*), bound to Reactant I via Reactant II. $y + z$ is the number of moles of Reactant II bound to Reactant I.

30

Complex in the detection zone:
Matrix (-Reactant I)$_{x-z-y}$ (-Reactant I --- Reactant II)$_z$ (-Reactant I --- Reactant II--- analyte --- Reactant*)$_y$


35  Simultaneous variants:
$m = 2$: The same as for protocol A with the exception that LZ$_2$R*/S is LZ$_2$R*/S/RII or that LZ$_1$B is LZ$_1$RII.

$m = 3$: LZ$_3$R*/S   LZ$_2$B   LZ$_1$RII   DZ   or

LZ$_3$R*/S   LZ$_2$RII   LZ$_1$B      DZ.

<u>Sequential variants</u>:

m = 2: The same as for protocol A with the exception that
LZ$_1$S is replaced by LZ$_1$S/RII.

m = 3: LZ$_3$R*      LZ$_2$B          LZ$_1$S/RII    DZ or

        LZ$_3$R*      LZ$_2$S          LZ$_1$RII      DZ or

        LZ$_3$R*      LZ$_2$S/RII      LZ$_1$B        DZ.

m = 4, 5, 6: In analogy with protocol A sequences with up
to 6 application zones for liquid may be considered.


C. Inhibition protocol:

Reactant I is an analyte analogue, firmly anchored to the
matrix, Reactant III exhibits biospecific affinity for the
analyte and Reactant* has biospecific affinity for Reactant
III. x = number of moles of Reactant I on the matrix. y =
number of moles of Reactant III (= number of moles of
Reactant*), bound to the matrix via Reactant I. The
conditions are selected so that y is a measure of the
amount of analyte in the sample.


Complex in the detection zone:
Matrix (-Reactant I)$_{x-y}$ (-Reactant I --- Reactant III ---
Reactant*)$_y$


<u>Simultaneous variants</u>:

m = 2:  LZ$_2$R*/RIII/S    LZ$_1$B      DZ.


<u>Sequential variants</u>:

m = 2:  LZ$_2$R*    LZ$_1$/RIII/S      DZ.

m = 3, 4 and 5: May be built up in analogy with protocol A.


D. Inhibition protocol

Reactant I exhibits biospecific affinity for both Analyte
and Reactant*. Reactant* is a soluble analyte analogue. x +
y is the number of moles of Reactant I on the matrix, x and
y are the number of moles of Reactant* and Analyte,
respectively, being bound to the matrix.

Complex in the detection zone:

Matrix (-Reactant I --- Reactant*)$_x$ (-Reactant I ---
Analyte)$_y$:

5

Simultaneous variant:

m = 2: LZ$_2$R*/S     LZ$_1$B     DZ


Sequential variant:

10   m = 2:  LZ$_2$R*     LZ$_1$S     DZ

m = 3, 4 and 5 may be built up in analogy with protocol A.


**Matrices**

The matrix defines the space in which the flow is

15   transported. The matrix may be the inner surface of a
simple flow channel (e.g. a capillary), the inner surface
of a porous matrix having a system of flow channels (porous
matrix) etc. extending through. For the sake of simplicity,
matrices, usable in this variant of the invention, will be

20   called flow matrices. Porous matrices may exist in the form
of monoliths, sheets, columns, membranes, single flow
channels having capillary dimensions or aggregated systems
of such flow channels etc. They may also exist in the form
of particles packed in column casings, compressed fibres

25   etc. The inner surface of the matrices, i.e. the surface of
the flow channels, should be hydrophilic, such that aqueous
media (usually water) may be absorbed and transported
through the matrices. The smallest inner dimension of the
flow channels should be sufficiently large for allowing

30   transport through the matrix of the reactants being used.
The rule of thumb is that suitable matrices are selected
among those having flow channels with the smallest inner
dimension (often as a diameter for round channels) in the
interval 0.4-1000 μm, preferably 0.4-100 μm if the matrix

35   exhibits a system of mutually communicating flow channels.
Flow channels having a smallest inner dimension in the
upper part of the interval 0.4-1000 μm are primarily of

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interest for simple unbranched channels, through which flow
is driven by an externally imposed pressure or suction.

Relevant matrices are often built up from a polymer, e.g.
5   nitrocellulose, nylon etc. The material in the matrix as
well as the physical and geometrical design of the flow
channels may vary along the flow depending on what a
certain part of the matrix is to be used for (Pharmacia AB
WO 96/22532; Medix WO 94/15215).
10

**Detection zone**

In the detection zone, Reactant I is firmly anchored to the
matrix with bonds not allowing unintentional transport of
Reactant I under the test conditions. Attachment of
15   Reactant I to the matrix may be covalent, via physical
adsorption, via biospecific affinity etc. Like prior art in
this field the invention may utilize combinations of
binding types, e.g. covalent binding to the matrix of a
biospecific affinity reactant directed to Reactant I. In
20   particular may be mentioned a matrix exhibiting a
physically adsorptively or covalently bound member of a
specific binding pair (reactant pair) in combination with
Reactant I coupled or conjugated to the other member of the
specific binding pair, or a matrix exhibiting a similarly
25   bound antibody directed to Reactant I. As examples of
specific binding pairs may be mentioned immunological
binding pairs, such as antigen-antibody and hapten-
antibody, biotin-avidin or -streptavidin, lectin-sugar,
hormone-hormone receptor, nucleic acid duplex. If reactant
30   I binds to the matrix via another reactant according to the
above, Reactant I need not be immobilized in the matrix but
may either be movably (diffusively) predeposited in the
matrix in an area or zone that is separated from the
detection zone, or it may be added together with or
35   separately from the sample. Anchoring of Reactant I to the
matrix may be achieved via particles having been deposited
in/on the matrix and to which Reactant I is covalently,
physically adsorptively or biospecifically etc. bound. The

particles attach to the matrix either because their size
has been selected such that they cannot be transported
through the matrix or via physical adsorption. See inter
alia Abbott/Syntex US 4,740,468; Abbott EP 472,376;
5  Hybritech EP 437,287 and EP 200,381; Grace & Co. EP
420,053; Fuji Photo Film US 4,657,739; Boehringer Mannheim
WO 94/06012. For the invention the latter variant with
smaller particles adsorbing physically to the matrix has
been shown to be good.
10

In one and the same transport flow there may be several
detection zones (DZ1, DZ2 etc.). One or more of the
detection zones may relate to the same or different
analytes. If the analytes are different Reactant I is
15  usually different for each DZ.


**Analytically detectable reactant (Reactant*)**

In the invention Reactant* cannot be an analyte. Usually
analytical detectability is obtained because a natural
20  reactant, e.g. an antibody or an antigen or a hapten, is
provided with an analytically detectable group. Well known
examples of often used groups are enzymatically active
groups (enzyme, co-factor, co-enzyme, enzyme substrate
etc.), fluorogenic, chromophoric, chemiluminiscent,
25  radioactive groups etc. Groups being detected by means of a
biospecific affinity reactant usually also are referred to
this category, e.g. biotin, hapten, class-, subclass- and
species-specific determinants in immunoglobulins etc.


30  A preferred label group is particles optionally containing
any of the detectable groups above, such as fluorophoric
groups or chromogenic groups (coloured particles). Useful
particles often have a size in the interval of 0.001-5 µm.
The particles may have colloidal dimensions, so called sol
35  (i.e. usually spherical and monodisperse having a size in
the interval 0.001-1 µm). Especially metal particles (e.g.
gold sol), non-metal particles (e.g. $SiO_2$, carbon, latex

and killed erythrocytes and bacteria) may be mentioned.
Also particles of non-colloidal dimensions but with focus
on non-sedimenting capability have been used. These have
been more or less irregular and more or less polydisperse

5    (carbon particles < 1 μm; Pharmacia AB, WO 96/22532).


For particles as label group reference is made to Unilever
WO 88/08534; Abbott US 5,120,643; Becton Dickinson EP
284,232 among others.

10

In connection with the development that has led to the
present invention it was surprisingly found that good
results may be obtained if one simultaneously utilizes:

15   (a)   Reactant* with label particles according to the above
           as a detectable group, and


     (b)   a detection zone, in which Reactant I has been
           anchored to the matrix via particles substantially

20         having dimensions that would allow transport of the
           particles as such through the matrix.


We have achieved functioning systems, in which label
particles and anchoring particles substantially have the

25   same dimensions. This means with great probability that the
     label particles may be larger than the anchoring particles
     and vice versa, as long as they remain smaller than the
     flow channels defined by the matrix. The system may
     function with or without pre-deposition of Reactant* in the

30   intended application zone. This embodiment is part of an
     invention, described in our co-pending PCT application:
     "Analytical method using particles and test kit for
     performing the method" (based on SE 9704935-7). This
     separate patent application is incorporated herein by

35   reference.

## Analytes

The invention is primarily adapted for determination of
biospecific affinity reactants of the types initially
mentioned. The reactants may be a cell or a virus or a part
5   thereof. In particular antigen, such as an immunoglobulin
or an antibody may be mentioned. For immunoglobulins the
determination may relate to a certain Ig and/or certain Ig
subclass. For antibodies the determination may relate to a
certain specificity, optionally also the Ig class or the Ig
10  subclass of the antibody. Relevant Ig classes are IgA, IgD,
IgE, IgG and IgM. Relevant Ig subclasses are IgG1, IgG2,
IgG3 and IgG4.

In sandwich variants (according to protocols A and B,
15  above) the analyte may be an antibody, directed to an
allergen/antigen/hapten and be derived from a certain
species, a certain Ig class or a certain Ig subclass. In
this case Reactant* may be an analytically detectable
antibody directed to an epitope specific for the species,
20  Ig class or Ig subclass and with Reactant I (protocol A) or
Reactant II (protocol B) as the allergen/antigen/hapten.
Alternatively the reverse may be selected, i.e. Reactant*
is the allergen/antigen/hapten and Reactant I and Reactant
II, respectively, is the antibody, directed to the analyte.
25  When the analyte is an antigen in general, both Reactant I
and Reactant* may be antibodies, directed to the antigen,
in protocol A. For protocol B it is Reactant II and
Reactant* that are antibodies directed to the antigen.

30  Competitive variants are most interesting for low molecular
analytes. Illustrative examples are antigen and hapten. For
protocol C Reactant I may be the antigen or the hapten,
firmly anchored to the matrix, Reactant III may be an
antibody, directed to the antigen, and Reactant* may be an
35  antibody, directed to Reactant III. For protocol D Reactant
I may be an antibody directed to the analyte and Reactant*
may be the analyte labelled with an analytically detectable
group.

The method of the invention may be performed as part of diagnosing allergy or autoimmune disease.

5   For the inventors it has been of special interest to measure anti-allergen antibodies of IgE or IgG class, for the latter preferably with focus on any of the subclasses mentioned. Measuring of allergen-specific antibodies can be utilized when diagnosing IgE mediated allergy.

10

**Samples**

Relevant samples may be of biological origin, e.g. from different body fluids (whole blood, serum, plasma, urine, saliva, tear fluid, cerebrospinal fluid etc.), from cell

15  culture media, processing procedures in biotechnology, from tissue extracts, from food stuff, from the environment (environmental analysis samples) etc. The samples may be pretreated in order to fit e.g. the matrix, the test protocol involved etc.

20

**Calibrators**

Determination methods of the type that the invention is related to, involve that the detectable signal from the analytically detectable reactant (Reactant*) is measured

25  and the measured signal (sample value) is taken as a measure of the amount of analyte present in the sample. To transfer the measurement signal to actual amounts of analyte the signal is usually compared to the corresponding signal (calibrator value) of known standard amounts of

30  analyte (calibrators). In connection with the present invention a new calibrator system has been developed, which applied to the present invention constitutes a best embodiment.

35  This separate invention means that the used calibrator in advance has been anchored to a matrix (matrix calibrator), preferably of the same type as the one utilized for sample run. When measuring calibrator values, matrix calibrator is

allowed to bind to Reactant*, and then the measurement
signal from Reactant* is measured in a way known per se. By
utilizing different amounts of matrix calibrator a series
of calibrator values may be obtained corresponding to
5  different pre-determined amounts of analyte in sample
(standard amounts, dose-response curve, calibration curve).

Alternatively, instead a binder for the calibrator has been
anchored to the matrix, and calibrator is added at the
10  determination of calibrator value, optionally pre-deposited
in the matrix upstream of the calibrator zone(s) to be
dissolved by sample solution or buffer at the
determination. When a calibrator binder is bound to the
matrix, the calibrator may either be movably (diffusively)
15  pre-deposited in the matrix in a zone separated from the
detection zone, or be added together with or separately
from the sample. The calibrator binder is usually one
member of a specific binding pair (reactant pair), the
other member of the binding pair being coupled or
20  conjugated to the calibrator substance. As examples of such
specific binding pairs may be mentioned immunological
binding pairs such as antigen-antibody and hapten-antibody,
biotin-avidin or -streptavidin, lectin-sugar, hormone-
hormone receptor, nucleic acid duplex.
25

Applied to the present invention our new calibrator system
involves primarily that the transport flow passes one or
more zones with a calibrator, firmly anchored to the matrix
in the respective calibrator zone (CZ).
30

Anchoring of a calibrator to the matrix in a calibrator
zone may be performed according to the same principles as
for anchoring of Reactant I to a detection zone.

35  Calibrator zones should be located downstream of an
application zone for liquid, intended for transport of
Reactant*. In relation to the detection zone (DZ) the
calibrator zone is preferably located upstream.

Our invention relating to calibrators is described in
detail in our co-pending PCT application with the title
"Method using a new calibrator and a device and test kit
5  including the calibrator" (based on SE 9704933-2). This
application is incorporated herein by reference.


**A second main aspect of the invention**

The flow matrix according to the above containing two or
10  more application zones for liquid, optionally in the form
of a kit wherein the flow matrix is comprised together with
the analytically indicatable reactant, constitutes a second
main aspect of the invention.


15  The invention is illustrated in the following non-limiting
experimental part.


**EXAMPLE 1: SEQUENTIAL METHOD WITH THE ZONE SEQUENCE: $LZ_4B$,
$LZ_3R^*$, $LZ_2B$, $LZ_1S$, DZ. DETECTION OF hIgE IN TEST VARIANT
20  WITH CARBON PARTICLE CONJUGATE**

**Methods and materials**

<u>Adsorption of phenyldextran to polystyrene particles</u>:
Phenyldextran (substitution degree: 1 phenyl group on each
25  fifth monosaccharide unit = 20%, Mw dextran 40,000,
Pharmacia Biotech AB, Uppsala, Sweden) was adsorbed to

polystyrene particles (0.49 µm Bangs Laboratories, USA) by
incubations under stirring with phenyldextran dissolved in
deionized water to 1) 5 mg/ml, 10% particle suspension, RT
30  30-60 minutes; 2) 5 mg/ml, 5% particle suspension, RT 1 h;
3) 20 mg/ml, 1-2% particle suspension, RT 3 h or overnight.
The particles were subsequently washed twice with deionized
water. The particle suspensions were centrifuged between
each incubation and wash (12,100xg, 25 min, Beckman, J-21,
35  JA-20, 10,000 rpm). The particle suspension was finally
sonicated (Ultrasonic bath, Branson 5210, 5 min).

Coupling of anti-human IgE antibody (anti-hIgE) to
polystyrene particle: Anti-hIgE was coupled to
phenyldextran coated polystyrene particles with CDAP (1-
cyano-4-dimethylamino-pyridinium bromide (Kohn J and
5  Wilchek M, FEBS Letters 154(1) (1983) 209-210).

Desalting and change of buffer of anti-hIgE was performed
by gel filtration (PD-10, Pharmacia Biotech AB) in $NaHCO_3$,
0.1 M, pH 8.5. 2.3 g of polystyrene particles (coated with
10 phenyldextran according to above) in 2% solution in 30% (by
volume) acetone were activated with 17 ml CDAP (0.44 M) and
14 ml TEA (0.2 M triethylamine, Riedel-deHaën, Germany).
CDAP was added with stirring for 150 seconds and TEA for
150 seconds. The particles were washed with 30% (by volume)
15 acetone and centrifuged at 12,100xg (25 min, Beckman, J-21,
JA-20, 10,000 rpm). 17 of anti-hIgE was coupled to the
activated particles (2%, 0.15 g in 0.1 M $NaHCO_3$ pH 8.5) in
incubating with stirring overnight at +4°C. The particles
were centrifuged and decanted before deactivating with
20 glutamic acid 0.05 M and aspartic acid 0.05 M in 0.1 M
$NaCHO_3$ pH 8.5 when incubating with stirring overnight at
+4°C. Coupled particles were washed once with 0.1 M $NaHCO_3$,
0.3 M NaCl, pH 8.5, once with 0.1 M HAc, 0.3 M NaCl pH 5,
once with 0.1 M $NaHCO_3$, pH 8.5 and once with 20 mM borate
25 buffer pH 8.5.

The concentration of particles was determined
spectrophotometrically at $A_{600}$ nm with untreated particles
as reference. Concentration of coupled protein was
30 determined by having anti-hIgE present during the coupling
and cpm measurement.

Carbon particle conjugate (Reactant*): This was prepared by
anti-hIgE being adsorbed to carbon particles (sp100, < 1
35 μm, Degussa, Germany) according to WO 96/22532 (Pharmacia
AB). The final solution used in the flow matrix contained
400 μg of carbon particles per ml.

Deposition of anti-hIgE-coupled particles on membrane: On
nitrocellulose sheets (Whatman, 8 μm, length 5 cm and width
25 cm with polyester backing, anti-hIgE particles (prepared
5    according to the above) were deposited in a zone over the
width of the sheet (future detection zone) with Linear
Striper (IVEK Corporation). The flow was 1 μl/sec and 1
μl/cm. The particles were diluted in borate buffer (20 mM,
pH 8.5, Dextran T5000 4.2% w/w, sorbitol 5.8% w/w). The
10   amount of deposited anti-IgE antibody was about 1000 ng/cm.
The sheets were dried for 1 h at 30°C.


Zones for application of buffer, sample and carbon particle
conjugate: Well separated from the zone, containing
15   deposited material, 4 Inplastor strips (Mylar with glue on
one side, Gelman) with a width of 1 mm were placed in
parallel with the zone and parallel with each other at a
distance of 5 mm from each other (zone spacers). The
Inplastor strips thus defined four zones with a width of 5
20   mm. The sheets were cut perpendicularly relative to the
zone containing deposited material, to strips with a width
of 0.5 cm (the length of the strip then became 5 cm)
(Singulator: Matrix 1201 membrane cutter, Kinematic
automation). The final strips exhibited four parallel zones
25   (application zones) separated by Inplastor strips as zone
spacers and a separate zone with deposited anti-hIgE
antibody (detection zone). As a comparison strips without
zone spacers, i.e. without separated application zones were
produced.
30
Test methodology: Strips with separated application zones
were mounted on a plane holder. At the top (0.5 cm) of the
strip (and with the detection zone as the nearest zone) a
sucking membrane was placed (Whatman, 17 Chr, width 3 cm).
35   The holder also gave a constant pressure on the sucking
membranes. For simultaneous application of reagents to the
four subzones a 4 channel Multipipette (Labsystems) was

used. The multipipette was loaded so that serum sample (30
μl) was applied in the application zone nearest to the
detection zone in the order of buffer (30 μl), carbon
particle conjugate (30 μl) and buffer (30+30 μl) in the
5    respective upstream application zone. For sequential
application to the strips without zone spacers, 30 μl of
sample were first applied to the lower end of the strip.
After suction of sample volume, buffer (30 μl), carbon
particle conjugate (30 μl) and buffer (30+30 μl) were added
10   successively after suction. The carbon particle conjugate
was prepared according to above and suspended in buffer.
The buffer was $NaPO_4$ 0.1 M, BSA 3%, $NaN_3$ 0.05%, sucrose 3%,
sodium chloride 0.2%, phenyldextran 0.05%, bovine
gammaglobulin 0.05%, pH 7.5. The binding of the carbon
15   particle conjugate to the detection zone was quantitated by
measuring of absorbance (Ultroscan XL, Enhanced Laser
Densitometer, LKB). IgE with standard concentrations in
plasma environment (0; 0.5; 2; 10; 50, and 200 KU/L) was
used as samples.
20

**Results**

Having four application zones for liquid and simultaneous
addition, the liquids were migrating in the order of the
application zones, i.e. the sample being in the zone
25   nearest to the detection zone was migrating first, without
being mixed with the washing solution of the following
application zone, which solution in turn started migrating,
when the sample had been transported out of the application
zone for samples. Correspondingly the liquids of the
30   remaining zones were migrating sequentially without being
mixed.

**Table 1:** Analysis results from runs with sequential
addition in one zone and from simultaneous addition in four
35   subzones (buffer, analytically detectable reactant, buffer,
sample).

|  | Sequential addition in one zone | Simultaneous addition in 4 subzones |
|---|---|---|
| IgE KU/L | (Abs x1000) | (Abs x1000) |
| 0,5 | 0 | 12 |
| 2 | 312 | 207 |
| 10 | 1241 | 831 |
| 50 | 1921 | 1560 |
| 200 | 2115 | 2044 |

In **Table 1** is shown that the uptake decreases slightly for
5  strips with discrete application zones compared to when
addition is performed in one and the same zone. The
decrease is, however, marginal. Therefore the experiment
shows that generally the same result may be achieved if
simultaneous addition is performed to the zone sequence
10  $LZ_4B$, $LZ_3R*$, $LZ_2B$, $LZ_1S$ as if sample, Reactant* and buffer
are added sequentially to a common application zone.

If a firmly anchored anti-IgE antibody (Reactant I) is
replaced by an allergen a determination method for IgE
15  antibodies directed to the allergen is obtained.
Analogously, test systems related to antibodies of another
class/subclass and with another specificity may be
determined. Application zones for only buffer may be
omitted. For additional alternative test protocols and
20  analytes see above under the headings "Test protocols" and
"Analytes".

EXAMPLE 2: SEQUENTIAL METHOD WITH THE ZONE SEQUENCE: $LZ_4B$, $LZ_3R^*$, $LZ_2B$, $LZ_1S$, DZ. DETECTION OF hIgE IN TEST VARIANT WITH FLUORESCENT DETECTION CONJUGATE

5 **Methods and materials**

Coupling of anti-human IgE antibody (anti-hIgE) to a polystyrene particle: Anti-hIgE was coupled to polystyrene particles coated with phenyldextran (prepared according to Example 1) with CDAP (1-cyano-4-dimethylaminopyridinium

10 bromide) (Kohn J and Wilchek M, FEBS Letters 154(1) (1983) 209-210). Desalting and buffer change of anti-hIgE was performed by gel filtration (PD-10, Amersham Pharmacia Biotech AB) in $NaHCO_3$, 0.1 M, pH 8,5.

15 0.35 g of polystyrene particles (2% solution) were activated with 5.2 ml of CDAP (0.44 M) and 4.2 ml of TEA (0.2 M triethylamine, Riedel-deHaën, Germany). CDAP was added with stirring for 60 seconds and TEA for 120 seconds. A five times excess of ice-cooled deionised water was

20 added. The particles were centrifuged at 12,100xg (25 min., Beckman, J-21, JA-20, 10,1000 rpm). The resulting pellet was dissolved in ice-cooled, deionized water and washed once with ice-cooled deionized water and then centrifuged at 12,000 x g. 50 mg of anti-hIgE were coupled to the

25 activated particles (2%, 0.35 g in 0.1 M $NaHCO_3$, pH 8.5). Incubation with stirring was the performed for 1 hour at +4°C. After centrifugation, the particles were deactivated with glutamic acid, 0.05 M, and aspartic acid, 0.05 M in 0.1 M $NaHCO_3$, pH 8.5. Incubation and stirring was then

30 performed overnight at +4°C. Coupled particles were washed twice with 20 mM borate buffer, pH 8.5, whereupon the particle concentration was determined spectrophotometrically at $A_{600nm}$ with untreated particles as reference. Coupled protein concentration was determined

35 by having radioactive anti-hIgE present during the coupling.

<u>Coupling of anti-hIgE antibodies to detection particles</u>:
Antibodies to hIgE cleaved with pepsin to fab'2 fragments
were coupled to fluorescent polystyrene particles having
aldehyde groups on their surface (Molecular Probes C-17177

5   TransFluoSphere, aldehyde-sulphate microspheres, 0.1 μm,
633/720, 2% solids). 23 mg of antibody were then coupled to
66 mg of particles in 50 mM $NaPO_4$, pH 6.5, overnight at
room temperature, whereupon 205 μL of $NaCNBH_4$ (5 M) were
added to reduce the coupling for 3 hours at room

10  temperature. Centrifugation was performed at 20,800 x g for
50 minutes (50 minutes in Eppendorf 5417R, 14,000 rpm), and
deactivation in glutamic acid, 0.05 M, and aspartic acid,
0.05 M, in deionized water, pH 6.5, was then performed
overnight with stirring at room temperature. After

15  centrifugation again at 20,800 x g, blocking was performed
with 0.2% BSA in 50 mM $NaPO_4$, pH 7.4, with 0.05% $NaN_3$ and
incubation was carried out overnight at +4°C.
Centrifugation was then performed again at 20,800 x g and
washing was performed twice with blocking buffer which was

20  then used also for storage. The particle concentration was
determined in a fluorimeter (Perkin-Elmer LS50B) with a
standard curve prepared with the original particle. Coupled
protein concentration was determined by having radioactive
hIgE present during the coupling.

25

<u>Deposition of anti-hIgE-coupled particles on membrane and
application zones</u>: Was performed according to Example 1,
except that the Inplastor strips were replaced by strips of
adhesive tape (2 mils clear polyester Arcare with glue on

30  one side).

<u>Test methodology</u>: Strips with spaced application zones were
mounted to an inclined plane, about 16°, in a holder. At
the top (0.5 cm) of the strip (and with the detection zone

35  as the nearest zone), two sucking membranes (Whatman, 17
Chr, width 3.4 cm) were placed on top of each other. The
holder also exerted a constant pressure on the sucking

membranes. For simultaneous application of reagents to the
four subzones, a multichannel Finn pipette (Labsystems) was
used. The multipipette was charged so that serum sample (30
µL) was applied to the application zone nearest to the

5    detection zone in the order of buffer (15 µL), fluorescent
particle conjugate (30 µL) and buffer (30 + 30 µL) in the
respective application zone located upstream. For
sequential application to the strips without zone spacer,
30 µL of sample were first applied to the lower end of the

10   strip. After sucking of the sample volume, buffer (15 µL),
detection particle conjugate (30 µL) and buffer (30 + 30
µL) were successively added after sucking. The particle
conjugate was suspended in assay buffer consisting of $NaPO_4$
0.1 M, BSA 3 %, $NaN_3$ 0.05 %, sucrose 10 %, NaCl, 0.15 M,

15   bovine gammaglobulin 0.05 %, pH 7.5. The measurement of
time started with the application of the sample, and the
time until the last buffer had been sucked into the
membrane was noted. The binding of the fluorescent particle
conjugate to the detection zone was quantified by scanning

20   with a red diode laser (635 ± 5 nm). As sample were used
IgE standard concentrations in plasma environment (0, 0.5,
2, 10, 50 and 200 KU/L).


**Results:**

25   Precisely as in Example 1, the liquids migrated out of the
application zone in the existing order. The time for a
whole test with simultaneous application was about 20
minutes, and the time for a test with sequential
application was about 25 minutes.

30

**Table 2**: Analysis results from runs with sequential
application in one zone and from simultaneous application
in four subzones (buffer, analytically detectable reactant,
buffer, sample).

35

| KU/L | Simultaneous addition to four subzones | Sequential addition to one zone |
|------|----------------------------------------|--------------------------------|
| 0    | 0.048*                                 | 0.038*                         |
| 0.5  | 0.053                                  | 0.047                          |
| 2    | 0.085                                  | 0.074                          |
| 10   | 0.286                                  | 0.256                          |
| 50   | 1.334                                  | 1.291                          |
| 200  | 2.507                                  | 2.487                          |

* = scanning signal (Vmm)

Table 2 shows that the uptake is comparable for strips with
5   discrete application zones in comparison with addition to a
single zone. The experiment therefore shows that the same
result may be obtained if addition is made simultaneously
to the zone sequence $LZ_4B$, $LZ_3R*$, $LZ_2B$, $LZ_1S$ as if sample,
Reactant* and buffer are added sequentially to a common
10  application zone.

**EXAMPLE 3: SEQUENTIAL METHOD WITH THE ZONE SEQUENCE: $LZ_5B$,
$LZ_4R*$, $LZ_3B$, $LZ_2S$, $LZ_1B$, DZ. DETECTION OF BIRCH-SPECIFIC**
15  **hIgE IN TEST VARIANT WITH FLUORESCENT DETECTION CONJUGATE**

**Methods and materials**

Extraction of birch pollen allergen t3, Betula verucosa: 1
part (weight) of birch pollen (Allergon, Sweden) was
20  extracted with 10 parts (volume) of 0.1 M phosphate buffer,
pH 7.4. The extraction was continued for 2 hours on a
shaker table at +4 °C. The extract was centrifuged at 4000
rpm for 1.75 hours. After filtration, the solution was
applied to a PD-10 column (Pharmacia Biotech AB) and eluted
25  in 0.1 M $NaHCO_3$, pH 8.5. The t3 eluate (designated: t3
extract 1/14) was taken to amino acid analysis for
determination of the total protein content.

Coupling of birch pollen allergen to polystyrene particle:
t3 extract was coupled to phenyldextran coated polystyrene
particles (prepared according to Example 1) with CDAP. The
coupling was effected analogously with the coupling of
5  hIgE.

Polystyrene particles (2128 mg) coated with phenyldextran
in 30 % (by volume) acetone, 2 % particle suspension, were
activated with 954 mg of CDAP (100 mg/ml in 30 % acetone)
10  and 7.63 ml of 0.2 M triethylamine (TEA, Riedel-de Haen,
Germany). CDAP was added with stirring and TEA was added
dropwise for 90 seconds and with stirring for totally 120
seconds. The reaction was stopped by the addition of 30 %
acetone (4 times the volume) and centrifugation at 12,400 g
15  for 35 minutes. The particles were washed once with
deionized water.

640 ml of t3 extract 1/14 in 0.1 M $NaHCO_3$, pH 8.5, were
added to the activated particles, and coupling was
20  continued for 1 hour on a shaker table. After
centrifugation, the particles were deactivated with 0.05 M
aspartic acid and 0.05 M glutamic acid in 0.1 M $NaHCO_3$, pH
8.5. Incubation then took place on a shaker table overnight
at +4 °C. The particles were then washed twice with 50 mM
25  $NaPO_4$, 0.05 % $NaN_3$, pH 7.4. The particle concentration was
determined spectrophotometrically at 600 nm with uncoated
polystyrene particles as reference. t3-coupled polystyrene
particles were taken to amino acid analysis for
determination of the total protein content.
30

Deposition of t3-coupled polystyrene particles on a
membrane: To nitrocellulose sheets with polyester backing
(Whatman, 8 μm, 5 cm width) were applied zones of t3-
coupled particles diluted to 4 % particle content in 50 mM
35  $NaPO_4$, 10 % sucrose, 0.05 $NaN_3$, pH 7.4. The deposits were
dried for 1 hour at 30 °C.

Zones for application of buffer, sample and detection
particle conjugate: Five 1 mm wide strips of adhesive tape
(2 mils clear polyester, Arcare with glue on one side) were
placed well separated from the zone containing the
5    deposited material and in parallel with the zone at a
distance of 5 mm from each other. The tape strips thereby
defined five different 5 mm wide zones. The sheets were cut
perpendicularly to the zone containing deposited material
to strips having a width of 0.5 cm (the length of the strip
10   then became 5 cm) (Singulator: Matrix 1201 membrane cutter,
Kinematic automation). The final strips exhibited five
zones (application zones) separated by tape strips as zone
spacers and a separate zone with deposited birch pollen
(detection zone). Strips without zone spacers, i.e. without
15   separated application zones, were prepared as a comparison.

Test methodology: Strips with separated application zones
were mounted, and reagents were applied as in Example 2.
Buffer (20 µL) was applied to the zone closest to the
20   application zone, and then serum sample (30 µL), buffer (20
µL), detection particle conjugate (20 µL) and buffer (30 +
30 µL) in the respective application zone located upstream.
For sequential application to the strips without zone
spacer 20 µL of buffer were first applied to the lower end
25   of the strip, and after sucking in thereof, 30 µL of sample
were applied in the same position and then buffer (20 µL),
fluorescent particle conjugate (20 µL) and buffer (30 + 30
µL). Before all applications, the preceding application had
been sucked in by the strip. The detection particle
30   conjugate and the buffer were according to Example 2.

**Results:**

With the application zone consisting of five subzones and
with simultaneous addition thereto, it turned out that the
35   liquids, precisely as in the Examples above, migrated out
of the application zone in the existing order. The time

required for a whole test with simultaneous addition was
about 21 minutes, and the time needed for a whole test with
sequential addition was about 27 minutes.

5  **Table 3**: Analysis results from runs with sequential
addition in one zone and from simultaneous addition in 4
subzones (buffer, analytically detectable reactant, buffer,
sample).

|      | Simultaneous addition to 5 subzones | Sequential addition to 1 zone |
|------|-------------------------------------|-------------------------------|
| neg  | 0.067*                              | 0.058*                        |
| pos 1| 1.911                               | 2.608                         |
| pos 2| 0.299                               | 0.375                         |

10

* = scanning signal (Vmm)

Table 3 shows that the uptake is decreased to some extent
for strips having discrete application zones compared with
15  addition to a single zone. The decrease is, however,
marginal and is probably due to the fact that the flow rate
in simultaneous application was somewhat delayed. The
experiment therefore demonstrates that basically the same
results may be achieved for simultaneous application to the
20  zone sequence $LZ_5B$, $LZ_4R*$, $LZ_3B$, $LZ_2S$, $LZ_1B$ as if sample,
Reactant* and buffer are applied sequentially to a common
application zone.

25

## CLAIMS

1.     A method for determination of an analyte in a sample
in a flow matrix by use of a transport flow of one or more
5  biospecific affinity reactants, at least one of which is
analytically detectable (Reactant*) and one is firmly
anchored in the matrix (Reactant I), and the flow matrix
comprises:

10  A) an application zone for liquid (LZ), containing buffer
and sample and optionally one or more of the reactants, but
not Reactant I,

B) a detection zone (DZ) located downstream of LZ with the
15  firmly anchored reactant (Reactant I), and

C) optionally one or more zones in which any of the
reactants has been pre-deposited,

20  wherein (i) the flow towards the detection zone is
initiated by addition of the liquid with sample in the
application zone LZS for transport of analyte and reactants
towards the detection zone (DZ), and (ii) the amount of
Reactant* bound to DZ is detected, the detected amount
25  being related to the amount of analyte in the sample,
**characterized** in that

I. the flow matrix comprises at least two application zones
for liquid arranged substantially adjacent to each other:
30

$LZ_m$  .  .  . $LZ_n$  .  .  . $LZ_1$                 DZ
---------------------------------------------------->

wherein

35

a) $LZ_n$ is an application zone for liquid, and n is the
position of the application zone $LZ_n$,

b) m is the total number of application zones in which
flow is initiated (m ≥ 2),

c) one $LZ_n$ is an application zone for sample $(LZ_n,S)$ and
one $LZ_n$ is for Reactant* $(LZ_{n''}.R*)$ with $n'' \geq n'$,

d) --------------> is the direction of the flow, and

e) DZ is the detection zone, and

II.    flow is initiated by adding liquid to each zone
$LZ_m$ .  . $LZ_n$ .  . $LZ_1$ in such a way that $liquid_{n+1}$, added to
the application zone $LZ_{n+1}$, is transported through the
matrix immediately after $liquid_n$, added to the nearest
downstream application zone $LZ_n$.

2.    The method according to claim 1, **characterized** in
that n'' > n' (sequential variants regarding analyte and
Reactant*).

3.    The method according to claim 1, **characterized** in
that n'' = n' (simultaneous variants regarding analyte and
Reactant*).

4.    The method according to any of the claims 1 - 3,
**characterized** in that Reactant* is pre-deposited in its
application zone $(LZ_{n''}.R*)$.

5.    The method according to any of the claims 1 - 4,
**characterized** in that $liquid_{n+1}$ is added to $LZ_{n+1}$ before or
substantially simultaneously with adding $liquid_n$ to $LZ_n$,
with the exception of n = m, which zone lacks the zone
$LZ_{n+1}$.

6.      The method according to any of the claims 1 - 5,
**characterized** in that $LZ_{n+1}$ finishes where $LZ_n$ starts, with
the exception of n = m, which zone lacks the zone $LZ_{n+1}$.

5   7.      The method according to any of the claims 1 - 6,
**characterized** in that application of liquid is performed
substantially simultaneously in all $LZ_m$ .   . $LZ_n$ .   . $LZ_1$.

8.      The method according to any of the claims 1 - 7,
10  **characterized** in that m ≤ 6; n' is 1, 2 or 3; n'' > n';
$LZ_{n'+1}$, $LZ_{n'+2}$, $LZ_{n'+3}$, $LZ_{n'-1}$, and $LZ_{n'-2}$ are application zones
for liquids intended for transport of Reactant* or other
reactant or buffer without reactant, as far as allowed by
m, n'' and n'.

15

9.      The method according to any of the claims 1 - 8,
**characterized** in that at least one of the zones
$LZ_m$ .   . $LZ_n$ .   . $LZ_1$ comprises a pad or material layer
applied on the flow matrix.

20

10.     The method according to any of the claims 1 - 8,
**characterized** in that the zones $LZ_m$   . $LZ_n$   . $LZ_1$ have
zone spacers between each other.

25  11.     The method according to any of the claims 1 - 10,
**characterized** in that the composition of transported
components from an application zone $LZ_n$ is not the same as
from the nearest adjacent application zone LZ, in which
flow is initiated, ($LZ_{n+1}$ and $LZ_{n-1}$, with the exception of n
30  = m and n = 1, which zones lack $LZ_{n+1}$ and $LZ_{n-1}$,
respectively).

12.     The method according to any of the claims 1 - 11,
**characterized** in that at least one reactant, other than
35  Reactant*, is pre-deposited in an application zone $LZ_{n...R}$
for liquid intended for transport of the reactant.

13.   The method according to any of the claims 1 - 12, **characterized** in that $m \leq 6$ and that $n'$ for the application zone for sample ($LZ_n.S$) is 1, 2 or 3.

14.   The method according to any of the claims 1 - 13, **characterized** in that Reactant* has biospecific affinity for the analyte so that Reactant* is incorporated into a complex Reactant'---Analyte---Reactant* in the detection zone in an amount related to the amount of analyte in the sample, in which complex Reactant' has biospecific affinity to the analyte and is

(a) Reactant I, or

(b) a reactant to which Reactant I exhibits biospecific affinity and which is transported from $LZ_n.S$ or from an application zone downstream of $LZ_n.S$.

15.   The method according to any of the claims 1 - 14, **characterized** in that the matrix comprises at least one calibrator zone (CZ), in which calibrator is bound to, or in advance has been bound to the matrix.

16.   The method according to claim 15, **characterized** in that the calibrator zone or zones (CZ) have a binder for the calibrator firmly anchored in the matrix, the calibrator optionally being pre-deposited in the matrix upstream of the calibrator zone or zones.

17.   The method according to any of the claims 1 - 16, **characterized** in that

a. the analyte is chosen among antigens generally, and
b. the method is performed as part of diagnosing allergy or autoimmune disease.

18.    A device for determination of an analyte in a sample
in a flow matrix by use of a transport flow of one or more
biospecific affinity reactants, at least one of which is
analytically detectable (Reactant*) and one is firmly

5   anchored in the matrix (Reactant I), said device comprising
a flow matrix having:


A) an application zone for liquid (LZ), containing buffer
and sample and optionally one or more of the reactants, but

10   not Reactant I,


B) a detection zone (DZ) located downstream of LZ with the
firmly anchored reactant (Reactant I), and


15   C) optionally one or more zones in which any of the
reactants has been pre-deposited,


**characterized** in that


20   the flow matrix comprises at least two application zones
for liquid arranged substantially adjacent to each other:


     $LZ_m$  .  .  .  $LZ_n$  .  .  .  $LZ_1$        DZ

     --------------------------------------------------->

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wherein


a) $LZ_n$ is an application zone for liquid, and n is the
position of the application zone $LZ_n$,

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b) m is the total number of application zones in which
flow is initiated (m ≥ 2),


c) one $LZ_n$ is an application zone for sample ($LZ_n$,S) and

35   one $LZ_n$ is for Reactant* ($LZ_{n''}$.R*) with n'' ≥ n',


d) -------------> is the direction of the flow, and

e) DZ is the detection zone.

19.  The device according to claim 18, **characterized** in
that n'' > n' and that the device is intended for
sequential transport of analyte and Reactant*.

20.  The device according to claim 18, **characterized** in
that n'' = n' and that the device is intended for
simultaneous transport of analyte and Reactant*.

21   The device according to any of the claims 18 - 20,
**characterized** in that Reactant* is pre-deposited in its
application zone $(LZ_{n'}.R*)$.

22.  The device according to any of the claims 18 - 21,
**characterized** in that $LZ_{n+1}$ finishes where $LZ_n$ starts, with
the exception of n = m, which zone lacks the zone $LZ_{n+1}$.

23.  The device according to any of the claims 18 - 22,
**characterized** in that m ≤ 6; n' is 1, 2 or 3; n'' > n';
$LZ_{n'+1}$, $LZ_{n'+2}$, $LZ_{n'+3}$, $LZ_{n'-1}$, and $LZ_{n'-2}$ are application zones
for liquids intended for transport of Reactant* or other
reactant or buffer without reactant, as far as allowed by
m, n'' and n'.

24.  The device according to any of the claims 18 - 23,
**characterized** in that the zones $LZ_m$  .  $LZ_n$  .  $LZ_1$ have
zone spacers between each other.

25.  The device according to any of the claims 18 - 23,
**characterized** in that at least one of the zones
$LZ_m$ .  . $LZ_n$ .  . $LZ_1$ comprises a pad or material layer
applied on the flow matrix.

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26.    The device according to any of the claims 18 - 25, **characterized** in that at least one reactant, other than Reactant*, is pre-deposited in an application zone $LZ_{n}...R$ for liquid intended for transport of the reactant.

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27.    The device according to any of the claims 18 - 26, **characterized** in that $m \leq 6$ and that n' for the application zone for sample $(LZ_{n}.S)$ is 1, 2 or 3.

10    28.    The device according to any of the claims 18 - 27, **characterized** in that the detection zone DZ comprises firmly anchored Reactant I, and that a reactant to which Reactant I exhibits biospecific affinity optionally is pre-deposited in $LZ_{n},S$ or in an application zone downstream of

15    $LZ_{n},S$.

29.    The device according to any of the claims 18 - 28, **characterized** in that the flow matrix comprises at least one calibrator zone CZ, in which a calibrator or a binder

20    for the calibrator is firmly anchored in the matrix.

30.    The device according to claim 29, **characterized** in that the calibrator zone or zones (CZ) have a binder for the calibrator firmly anchored in the matrix, and that

25    calibrator optionally is pre-deposited in the matrix upstream of the calibrator zone or zones.

31.    The device according to any of the claims 18 - 30, **characterized** in that the device is intended for diagnosing

30    allergy or autoimmune disease.

32.    A test kit, **characterized** in that the kit comprises (i) a device according to any of claims 18. - 29 and (ii) Reactant*.

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33.    The test kit according to claim 32, **characterized** in
that the kit additionally comprises (iii) a calibrator when
a binder for the calibrator is firmly anchored in the
matrix.

# INTERNATIONAL SEARCH REPORT

International application No.

**PCT/SE 98/02463**

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC6: G01N 33/53**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**IPC6: G01N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

**SE,DK,FI,NO classes as above**

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**WPI, EPODOC, TXTE**

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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| A | WO 9202818 A1 (PURDUE RESEARCH FOUNDATION), 20 February 1992 (20.02.92) -- | 1-33 |
| A | WO 9114942 A1 (PACIFIC BIOTECH, INC.), 3 October 1991 (03.10.91) -- -------- | 1-33 |

☐ Further documents are listed in the continuation of Box C.    ☒ See patent family annex.

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 24 March 1999 | 28 -03- 1999 |

| Name and mailing address of the ISA/ | Authorized officer · |
|---|---|
| Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86 | Hampus Rystedt Telephone No. + 46 8 782 25 00 |

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02/03/99

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